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Introduction

Dimethylsulfoniopropionate: an interesting precursor of natural flavor compounds ?

Production of natural flavors. Flavor compounds play an important role in the manufacturing of food. Traditionally, mixtures of synthetically produced flavors are used for flavoring of, for example, candy, snacks, and certain dairy products. Nowadays, the use of naturally produced flavors is becoming increasingly important, because consumers tend to prefer natural compounds for health reasons (Armstrong and Yamazaki 1986). With the aid of extraction techniques it is possible to obtain flavors directly from plant material, but these methods are time-consuming and expensive, because the most interesting flavors are present in only very low concentrations. A more recent method to produce flavors is based on a biotechnological approach where natural precursors, isolated mainly from plant material, can be converted in a bioreactor with the aid of enzymes and/or microorganisms to the desired flavor (Tyrell 1990). The difference in price between a natural and a synthetic compound can be very large; synthetic vanillin costs $ 12 /kg, when extracted from vanilla pods the price is $ 4000 /kg (Feron et al. 1996). This large difference in price clearly demontrates that other methods for the production of natural flavors are needed. Some examples of important flavors that can be produced with a biotechnological approach are given below.

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is one of the most common flavor chemicals; it is used in a broad range of flavors. The consumption of vanillin is estimated to be 12000 tons/year; only 50 tons are in the form of natural vanillin from the pods of *Vanilla planifolia* (Lomascolo et al. 1999). A lot of research is done in order to produce vanillin that can be labelled as natural. Production of vanillin is possible from ferulic acid or vanillic acid by several fungi; the preferred organism *Pycnoporus cinnabarinus* produces only 40 –90 mg vanillin/l in 5 to 7 days (see, for example, Gross et al. 1993 or Lesage-Meessen et al. 1999). Production of vanillin is also possible from eugenol, a cheap constituent of clove oil, for example by a *Pseudomonas* strain (Washisu et al. 1993).

Various conversions are known which lead to γ-decalactone (the peach-like flavor) with the aid of fungi or bacteria (Krings and Berger 1998). A number of patents describe the fermentation of castor oil or other substrates to γ-decalactone (see, for example, Farbood and Willis 1985; Farbood et al. 1990; Cheetham et al. 1993; Kumin and Munch 1998). Castor oil from seeds of *Ricinus communis* in which approximately 85% of the fatty acids are ricinoleic acid, one of the most important precursors, is a good substrate for *Yarrowia lipolytica* (Farbood and Willis 1985). Also other microorganisms, such as *Candida, Sporobolomyces* and *Rhodotorula* species carry out this fermentation in rich media and produce γ-decalactone in yields ranging from approximately 0.3 – 1.0 g γ-decalactone/l in 7 days (Cheetham et al. 1993). Fermentation of ricinoleic acid to γ-decalactone by *Saccharomyces cerevisiae* results in 2.0-3.75 g γ-decalactone per kg fermentation broth (Boog et al. 1998). Unlike some of the other organims mentioned, *Saccharomyces cerevisiae* is an acceptable organism for making food grade products.

Sulfur-containing flavors and dimethylsulfoniopropionate. There are many examples of sulfur-containing flavors present in food. Often, these sulfur-containing compounds are present as natural flavors in food and contribute already in low concentrations to its total flavor. Dimethylsulfide (CH_3-S-CH_3) and methanethiol (CH_3-SH) are naturally present in several types of food or drinks, like raw milk (Patton et al. 1956; Law and Sharpe 1978), beer (Walker and Simpson 1993), cheese, garlic, and tea (Schreier and Drawert 1975). High concentrations of dimethylsulfide results in an unpleasant, cabbage like odor. Esters of methylthiopropionate (MTPA; or methylmercaptopropionate) and mercaptopropionate (MPA; Fig. 1) can be used as a flavoring ingredient (Hansen and van der Maarel 1998; Hansen et al. 1998). For example, ethyl-mercaptopropionate provides a pleasant grape flavor (Kolor 1982). The use of the methyl ester of MTPA as flavoring ingredient results in a fruity flavor (van der Maarel and Hansen 1998). Methyl and ethyl esters of MTPA naturally occur in pineapple fruits. These compounds can be used in, for example, baked goods, frozen dairy, meat products, soft candy and non-alcoholic beverages in low ppm concentrations (Fenaroli 1995).

An interesting precursor for several sulfur-containing flavors is beta-dimethylsulfoniopropionate (DMSP; formerly known as dimethyl-beta-propiothetin). DMSP is mainly present in the marine environment where it acts as an osmolyte in many algae (Reed 1983; Keller 1988) and in some higher plant species (see, for example, Dacey et al. 1987). DMSP can be used as feeding stimulator for fishes and shellfishes to promote their appetites (Nakajima and Itoh 1992). Tatsuya and coworkers (1988) suggested that DMSP, obtained from marine algae with a high DMSP content, can be used as a natural flavor precursor for food and drink. The major reason for scientific interest in DMSP during the past 15 years stems from an interest in dimethylsulfide, which is one of the products after DMSP is cleaved (Fig. 1). In the last five years, this interest in DMSP has resulted in two symposia (The First International Symposium on DMSP and Related Sulfonium Compounds, held in Mobile, Alabama, USA in June 1995, and the Second International Symposium on Biological and Environmental Chemistry of DMS(P) and related compounds, held in Haren, The Netherlands in August 1999), and in a number of Ph.D projects at the University of Groningen. Examples are: "Anaerobic microbial degradation of methylated sulfur compounds", by M.J.E.C van der Maarel (1996), "The smell of the sea. Production of dimethylsulfoniopropionate and its conversion into dimethylsulphide by the marine phytoplankton genus *Phaeocystis*", by J. Stefels (1997), and "Microbial production and consumption of dimethyl sulfide (DMS) in intertidal sediment ecosystems", by H.M. Jonkers (1999).

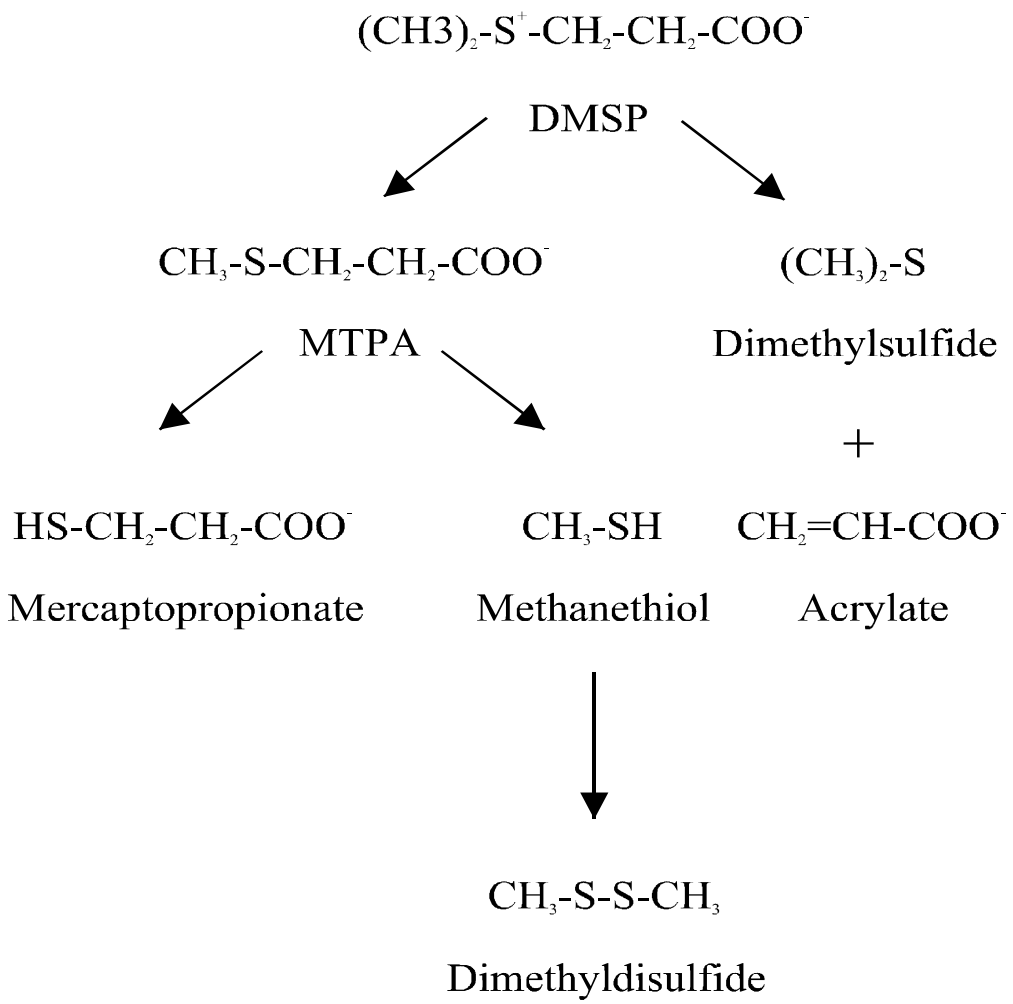

Fig. 1 Pathways for the microbial degradation of dimethylsulfoniopropionate

DMS(P) and the environment. Almost thirty years ago it became clear that dimethylsulfide is quantitatively the most important compound involved in the transport of sulfur from the ocean to land; it therefore plays an important role in the global sulfur cycle (Lovelock et al. 1972). This was not the only reason for the increased interest in dimethylsulfide. It has also been suggested that the emision of dimethylsulfide has an important effect on the climate of the earth (Bates et al. 1987; Charlson et al. 1987). When dimethylsulfide reaches the atmosphere it can be oxidized by radicals, such as hydroxyl radicals, resulting in dimethylsulfoxide and acidic products (methanesulfonic acid, sulfur dioxide, sulfuric acid) which act as cloud condensation nuclei and decrease the amount of sunlight reaching the earth's surface. This directly affects the climate of the planet. Another important effect of the oxidation products is their contribution to the acidity of rain water (Charlson and Rodhe 1982).

Biosynthesis of DMSP. DMSP is present mainly in the marine environment where it acts as an osmolyte in many different macro- and microalgae (Reed 1983; Keller 1988), but also in some higher plant species, such as *Spartina anglica* (van Diggelen et al. 1986), *Spartina alterniflora* (Dacey et al. 1987), *Wollastonia biflora* (Hanson et al. 1994) and sugarcane (Paquet et al. 1994). The pathway of DMSP formation in algae was elucidated recently by Gage et al. (1997). With in vivo isotope labelling, DMSP formation from methionine was shown to occur in the green

macroalga *Enteromorpha intestinalis*. Methionine conversion involved the following steps: transamination, reduction, S-methylation, and oxidative decarboxylation. The intermediates in this pathway to DMSP are: 4-methylthio-2-oxobutyrate, 4-methylthio-2-hydroxybutyrate and 4-dimethylsulfonio-2-hydroxybutyrate (Fig 2A). The compound 4-dimethylsulfonio-2-hydroxybutyrate was also identified in three diverse phytoplankton species, which indicates that the same pathway exists in other algal classes.

In plants the biosynthesis of DMSP is different (Fig 2B). In *Wollastonia biflora* leaves the first step was a methylation of methionine to *S*-methylmethionine catalyzed by the enzyme *S*-adenosylmethionine:methionine *S*-methyltransferase (Hanson et al. 1994). *S*-Methylmethionine is converted further in one step to DMSP-aldehyde, via a coupled transamination-decarboxylation reaction (Rhodes et al. 1997). In *Spartina alterniflora*, *S*-methylmethionine is not degraded in one step to DMSP-aldehyde, but via the intermediate DMSP-amine (Kocsis et al. 1998). Evidence that DMSP-aldehyde was an intermediate in the biosynthesis of DMSP in *Wollastonia biflora* was obtained by James and coworkers (1995) in three ways. In pulse-chase experiments with [^{35}S]methylmethionine, DMSP-aldehyde labeled as an intermediate and other possible intermediates did not. When adding [^{35}S]methylmethionine with other unlabeled compounds, only DMSP-aldehyde promoted [^{35}S]DMSP-aldehyde formation, while the other possible intermediates had no trapping effects. Plants without DMSP accumulation did not form [^{35}S]DMSP-aldehyde from [^{35}S]methylmethionine.

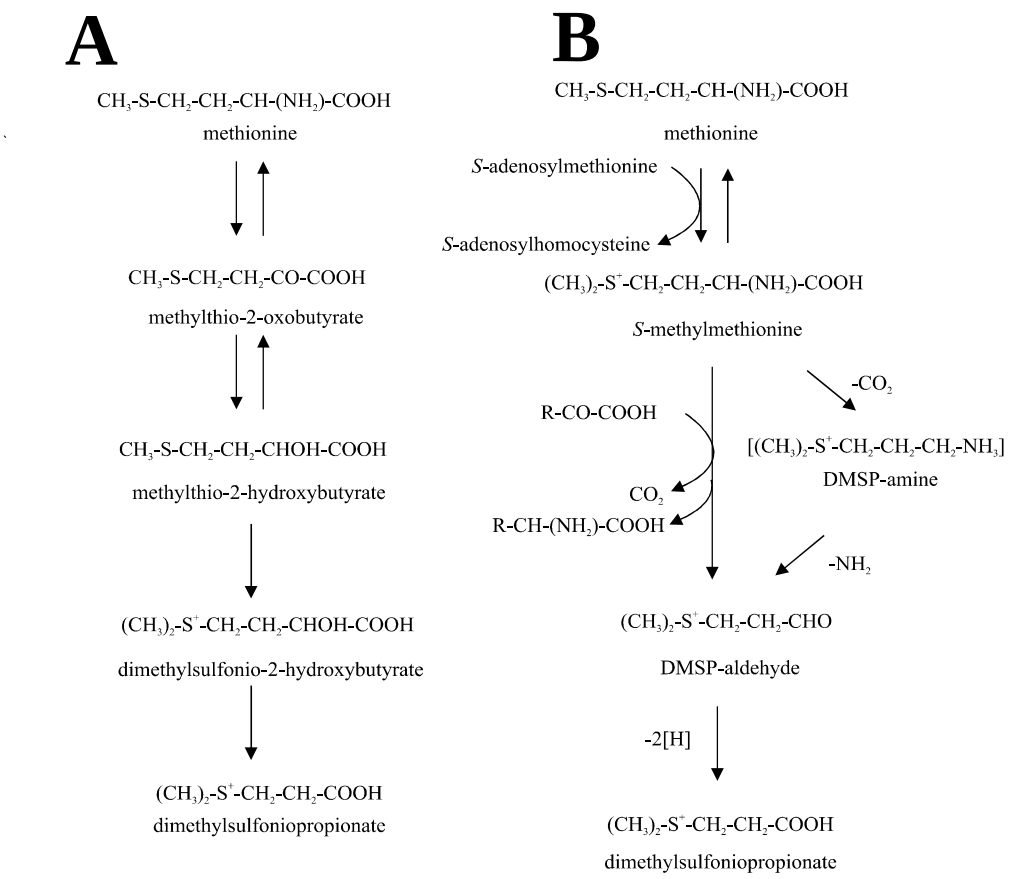

Fig. 2 Biosynthesis of dimethylsulfoniopropionate in *Enteromorpha intestinalis* (A) and *Wollastonia biflora* and *Spartina alterniflora* (B). Adapted from McNeil et al. (1999); see text for details

Microbial degradation of DMSP

DMSP is released from algae by lysis due to senescence (Stefels and van Boekel 1993), grazing (Dacey and Wakeham 1986) or viral activity (Bratbak et al. 1993, 1995). Most likely, a major part of the DMSP is degraded by bacteria. Kiene and coworkers were the first to describe the degradation pathways for DMSP in anoxic marine sediments (Kiene and Taylor 1988a and b; Kiene et al. 1990), and a few years later the same pathways turned out to be true for oxic environments as well (Taylor and Gilchrist 1991). The two different routes for degradation of DMSP are shown in Fig. 1. One possible route is the cleavage of DMSP, the other route involves a demethylation step. The microorganisms involved in the degradation of DMSP will be described in more detail below.

Cleavage of DMSP. The cleavage of DMSP to dimethylsulfide, acrylate and a proton (Fig. 1) is catalyzed by DMSP lyase (dimethylpropiothetin dethiomethylase EC 4.4.1.3). This enzyme has been detected in both aerobic and anaerobic bacteria, algae and even some fungi (see, for example, Ledyard et al. 1993; van der Maarel et al. 1996c; Stefels and Dijkhuizen 1996; Bacic et al. 1998). Examples of aerobic organisms catalyzing this cleavage reaction are numerous: they include strain LFR (Ledyard et al. 1993), *Pseudomonas doudoroffi*, *Alcaligenes* sp. strain M3A (De Souza and Yoch 1995a), strain MD 14-50 (Diaz et al. 1992), strain ML-D (Diaz and Taylor 1996), *Fusarium lateritium* (Bacic and Yoch 1998). Recently, Gonzalez et al. (1999) showed that fifteen marine, aerobic strains were able to produce DMS from DMSP, including isolates that were obtained without any selection for sulfur metabolism.

Already in 1962, Wagner and Stadtman demonstrated the anaerobic cleavage of DMSP by *Clostridium propionicum* (Wagner and Stadtman 1962). Recently, a *Desulfovibrio acrylicus* strain was isolated in our laboratory; this organism reduces sulfate and acrylate and is able to cleave DMSP very rapidly. It did not ferment the acrylate produced from the cleavage reaction, but used it as an electron acceptor (van der Maarel et al. 1996c).

Demethylation of DMSP. The initial demethylation of DMSP results in MTPA (Fig. 1). The MTPA can be demethylated further to mercaptopropionate, or the MTPA can be converted to methanethiol and further to dimethyldisulfide. Demethylating bacteria have been isolated from both aerobic (Taylor and Gilchrist 1991; Visscher and Taylor 1994; Diaz and Taylor 1996) and anaerobic (van der Maarel et al 1993, 1996b) environments.

Examples of aerobic organisms able to demethylate DMSP are strain DG-C1, strain MM-P, and *Alteromonas macleodii*. These bacteria produced methanethiol from DMSP (Taylor and Gilchrist 1991; Diaz and Taylor 1996; Ledyard et al. 1993). Strain BIS-6 demethylated DMSP stoichiometrically to mercaptopropionate (Visscher and Taylor 1994). Completely new was the observation from Gonzalez et al. (1999) that five marine bacterial strains were not only able to cleave the DMSP, but also possessed the demethylation pathway, with methanethiol as sulfur-containing end product. Kiene et al. (1999) showed that these bacteria, and also the DMSP-cleaving organisms isolated in that study, used DMSP and methanethiol as a source of sulfur for methionine and protein-sulfur.

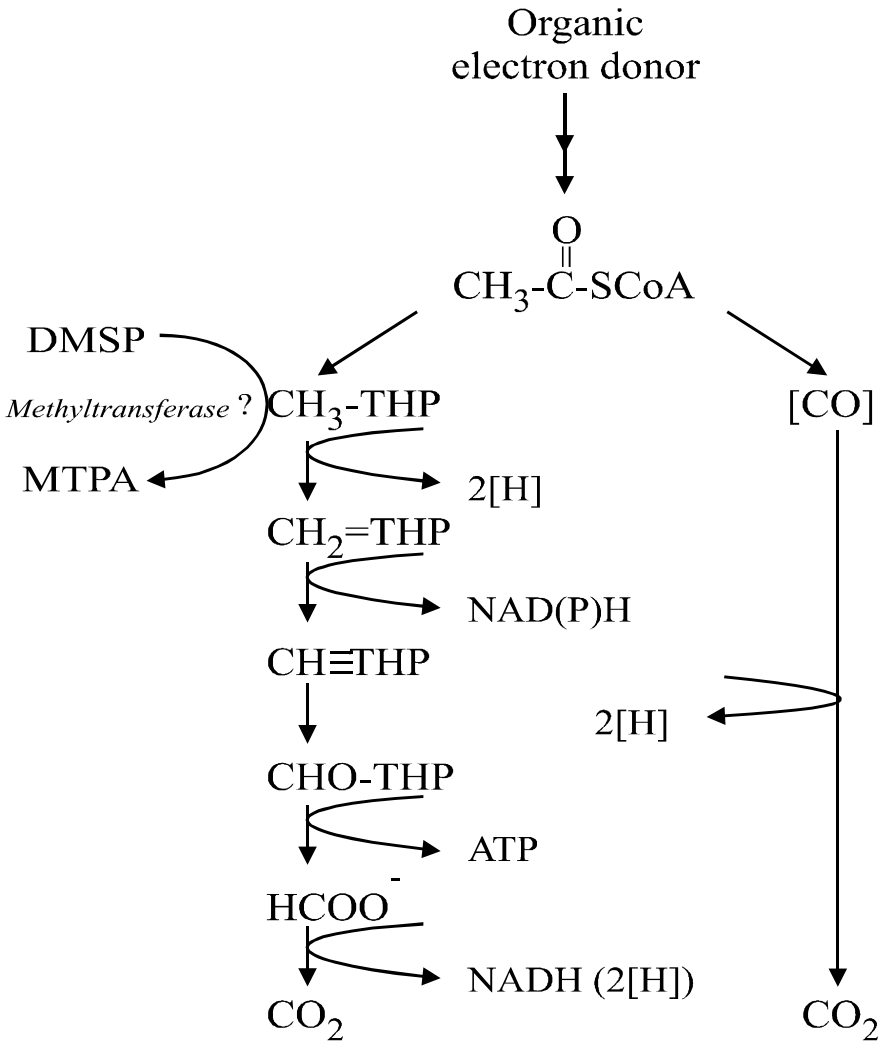

Fig. 3 Oxidative acetylCoA/CO dehydrogenase pathway used by sulfate-reducing bacteria such as *Desulfobacterium* and *Desulfotomaculum* (THP = tetrahydropterin, see Widdel and Hansen 1992)

Anaerobic demethylation of DMSP by a pure bacterial culture was demonstrated by van der Maarel et al. (1993). The marine sulfate-reducing bacterium *Desulfobacterium* strain PM4 oxidized one methyl group of DMSP and coupled this to the reduction of sulfate. The product of this demethylation reaction, MTPA, was not used by this strain. Several other marine sulfate-reducing bacteria belonging to the genus *Desulfobacterium* (or very similar to it) are able to demethylate DMSP stoichiometrically to MTPA, and in this way grow on the basis of the oxidation of the methyl group (van der Maarel et al. 1996b). Most likely, these bacteria channel the methyl group into the oxidative acetyl-CoA/CO dehydrogenase pathway, which is normally involved in the oxidation of acetyl-CoA (Fig. 3; van der Maarel et al. 1996b).

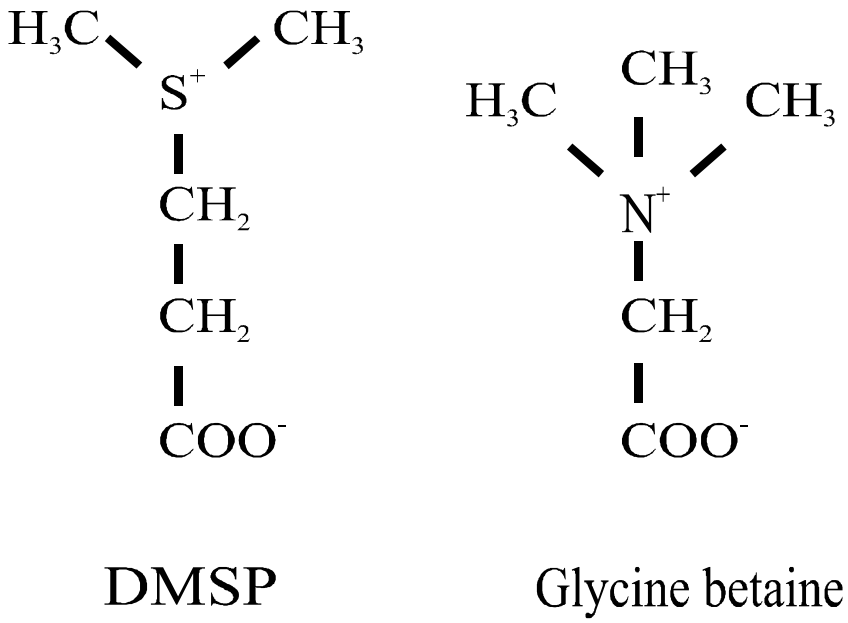

Fig. 4 Comparison of dimethylsulfoniopropionate and glycine betaine

Besides the already mentioned *Desulfobacterium* strain PM4, also *Db. niacini*, *Db. vacuolatum* and strain WN did not only demethylate DMSP, but also the N-containing structural analog of DMSP, glycine betaine (Fig. 4), which is a widely used compatible solute (Yancey et al. 1982). However, *Db. autotrophicum* grows on glycine betaine, but is unable to grow on DMSP (van der Maarel et al. 1996b). Acetogenic bacteria are obligate anaerobes that synthesize acetyl-CoA from C_1-compounds both for conservation of energy and for growth (Drake 1994). Generally, acetate is the main end product, but also longer fatty acids, like butyrate, can be produced (Fig. 5; more detailed information about acetogenic bacteria is described in the part dealing with the methyltransferases in acetogenic bacteria). Several acetogenic bacteria are able to grow on glycine betaine (see, for example, Heijthuijsen and Hansen 1990), but the ability of acetogens to demethylate DMSP was not known when this thesis project started. Kiene and Taylor (1988b) suggested that such acetogens might be involved in the demethylation of DMSP in anoxic sediments. Data from van der Maarel et al. (1996b) showed that the marine DMSP-demethylating sulfate-reducing bacteria, probably play an important role in this conversion in anoxic sediments due to their low K_m values for DMSP degradation and their high abundance. Whether acetogens play a major role in DMSP demethylation in marine, anoxic sediments is not known, but appears not very likely. Several acetogens, originating mainly from non-marine environments, are also able to demethylate DMSP to MTPA, but growth was not observed on this substrate and K_m values for DMSP degradation were rather high (van der Maarel et al. 1996b; chapter 3 in this thesis).

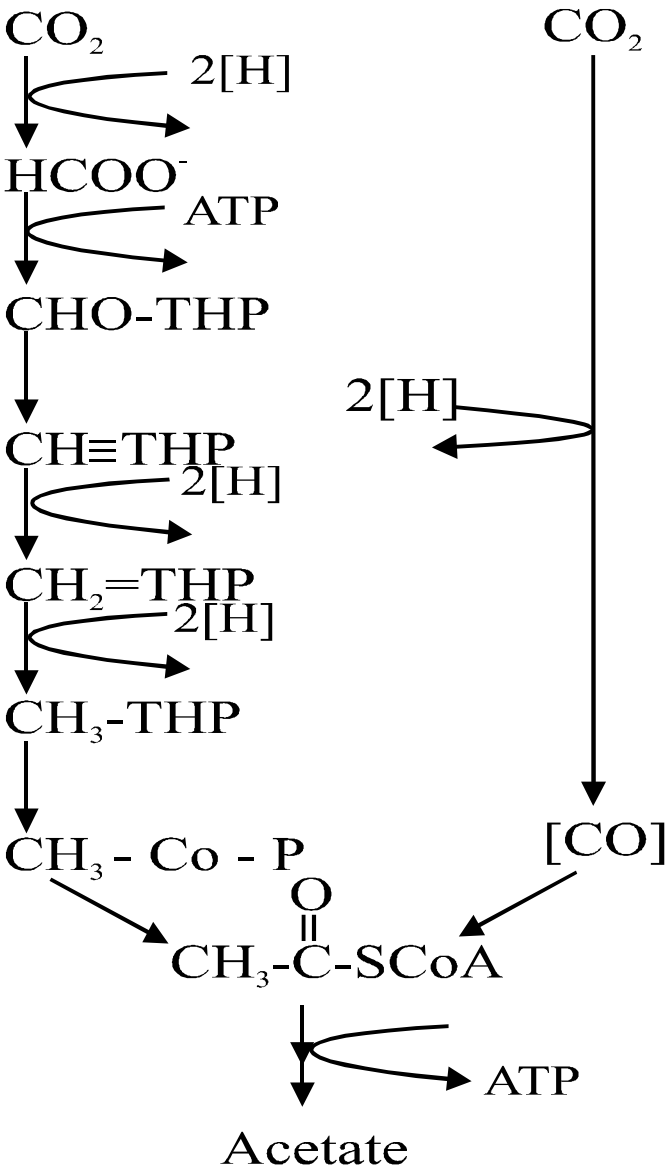

Fig. 5 Acetate synthesis from CO_2 by homoacetogenic bacteria (Acetyl CoA/ CO dehydrogenase pathway; THP = tetrahydropterin, CH_3-Co-P = methylated corrinoid/Fe-S protein); see Diekert and Wohlfarth 1994

Biochemical aspects of DMSP demethylation reactions

Until a few years ago, virtually nothing was known about the biochemistry of DMSP demethylation. This is somewhat surprising, because of the large amounts of DMSP produced in the marine environment. As shown above, the DMSP degraded by bacteria proceeds not only via an initial cleavage reaction, but is also possible via a demethylation pathway (Fig. 1). Isolation of bacteria growing aerobically on MTPA (and DMSP) was very easy (Taylor and Gilchrist 1991), and the authors concluded that the demethylation of DMSP to MTPA might be a significant process in the marine environment. The quantitative importance of the demethylating bacteria of the total DMSP degraders in the Caribbean Sea was demonstrated by Visscher et al. 1992). Estimated dimethylsulfide emissions from the oceans to the atmosphere are approximately 50 million tons S/year (Barnard et al. 1982, Malin 1996). Since not all DMSP is cleaved to dimethylsulfide and dimethylsulfide consumption seems to be a

very important factor (Kiene and Bates 1990), this implies that the flux of DMSP via the demethylation pathway must be large. DMSP lyases have been purified from a number of bacterial strains (de Souza and Yoch, 1995b; van der Maarel et al. 1996a). In contrast reports describing purified bacterial enzymes that catalyze the demethylation of DMSP are not available.

Possible DMSP demethylation reactions. Theoretically, aerobic or anaerobic demethylation of DMSP can be catalyzed by several enzymes.
(A) In aerobes a DMSP-oxidase may be involved, which results in the following reaction:
DMSP + O_2 + tetrahydrofolate $\rightarrow$ MTPA + H_2O_2 + methylenetetrahydrofolate

An example of an enzyme catalyzing such a type of reaction is sarcosine oxidase. The enzyme from *Corynebacterium* sp. P-1 catalyzes the oxidative demethylation of sarcosine which yields glycine, hydrogen peroxide and methylenetetrahydrofolate (Chlumsky et al. 1995). In the absence of tetrahydrofolate, formaldehyde is produced.
(B) A second possibility in aerobes is an oxygenase:
DMSP + O_2 + electron $donor_{reduced}$ $\rightarrow$ MTPA + H_2O + electron $donor_{oxidized}$ + HCHO (formaldehyde)

An example of an enzyme catalyzing such a type of reaction is dimethylsulfide monooxygenase. This enzyme catalyzes the oxidation of dimethylsulfide to methanethiol in *Hyphomicrobium* S. Most probably, formaldehyde is produced and assimilated by the serine pathway (de Bont et al. 1981).
(C) A third possibility is a DMSP dehydrogenase:
DMSP + electron $acceptor_{oxidized}$ + H_2O $\rightarrow$ MTPA + electron $acceptor_{reduced}$ + HCHO

The flavoprotein dimethylglycine dehydrogenase (EC 1.5.99.2) catalyzes such a type of reaction, where demethylation of dimethylglycine results in sarcosine formation (Frisell and MacKenzie 1962).
(D) A fourth possibility is a methyltransferase:
DMSP + methyl acceptor $\rightarrow$ MTPA + methylated methyl acceptor

Methyl acceptors that might play a role in this demethylation reaction are, for example, tetrahydrofolate (THF, also called tetrahydropteroylglutamate) and related compounds, such as tetrahydropterin (THP, similar to THF with 2 or more glutamate molecules), or homocysteine. During the thesis project we made considerable progress in understanding DMSP demethylation in certain anaerobes. Oxygen-dependent reactions are impossible in such organisms. We detected a DMSP-demethylating enzyme which belongs to the methyltransferases. Therefore, in the last part of this chapter we discuss methyltransferase reactions with THF or homocysteine as a methyl acceptor, and methyl transfer in methanogenic archaea and acetogenic bacteria.

Methyltransferases with tetrahydrofolate or homocysteine as methyl acceptor. THF is a methyl acceptor in trimethylsulfonium-THF methyltransferase in a Pseudomonas strain (Wagner et al. 1967). This enzyme catalyzes the demethylation of trimethylsulfonium chloride to dimethylsulfide, where THF acts as methyl acceptor.
Trimethylsulfonium + THF $\rightarrow$ dimethylsulfide + methyl-THF + H^+

This enzyme does not use DMSP or *S*-adenosylmethionine as methyl donor, and also important to mention for the remainder of this chapter, no vitamin B_{12} derivative is associated with the enzyme.

An example of an enzyme where homocysteine acts as a methyl acceptor is methionine synthase (EC 2.1.1.14). This reaction is important in the synthesis of methionine. Methionine synthase catalyzes the transfer of the methyl group from methyl-THF to homocysteine, which yields THF and methionine (see, for a recent review, Ludwig and Matthews 1997).

Methyl-THF + homocysteine → THF + methionine

Certain methionine synthases are corrinoid-dependent and others are corrinoid-independent (Stupperich 1993). Corrinoids are compounds containing cobalt and four reduced pyrrole rings. The best known example of this group is vitamin B_{12}. Over the years several different corrinoids have been isolated from methanogenic archaea and acetogenic bacteria (Stupperich et al. 1990).

Another example where homocysteine acts as a methyl acceptor is in the reaction catalyzed by the corrinoid independent betaine:homocysteine methyltransferase (EC 2.1.1.5):

Glycine betaine + homocysteine → dimethylglycine + methionine

This protein is found not only in bacteria (White et al. 1973; Smith et al. 1988), but also in eukarya (Garrow 1996). More than 40 years ago, it was reported that a thetin:homocysteine transmethylase from horse liver used besides dimethylacetothetin (modern name: dimethylsulfonioacetate) also glycine betaine and DMSP (Durell et al. 1957). Later it was confirmed by other researchers, that DMSP could serve as methyl donor for these enzymes. Physiological and biochemical evidence shows that in *Sinorhizobium meliloti* dimethylsulfonioacetate, the acetate analog of DMSP, is demethylated via the glycine betaine demethylating system, most likely a glycine betaine:homocysteine methyltransferase (Smith et al. 1988). DMSP, however, is not demethylated by *Sinorhizobium meliloti* and is used only as an osmoprotectant (Pichereau et al. 1998). Similarly, in extracts of *Pseudomonas denitrificans* glycine betaine and dimethylsulfonioacetate can function as methyl donors for homocysteine methylation whereas DMSP cannot (White et al. 1973).

Methyltransferases in methanogenic archaea. A great deal of research has been done to elucidate the mechanism of the methyl transfer reactions involved in the degradation of several methylated substrates in methanogenic archaea. Methanogenic archaea are involved in the conversion of compounds such as methanol (Daas et al. 1996; Sauer et al. 1997), methylamines (Ferguson and Krzycki 1997; Asakawa et al. 1998; Wassenaar et al. 1998) and methylthiols (Tallant and Krzycki 1997) to methane. Corrinoid proteins play an important role in these methyl transfer reactions to coenzyme M. The conversion of methanol to methane in *Methanosarcina barkeri* proceeds via methyl-coenzyme M. Two methyltransferases play an important role (van der Meijden et al 1983). First, the corrinoid of methyltransferase 1 (methanol: 5-hydroxybenzimidazolylcobamide methyltransferase) is methylated by methanol. Next, the methyl group is transferred to coenzyme M by methyltransferase 2 (methylcobamide:coenzyme M methyltransferase). Several reviews deal with the methyl transfer reactions in methanogens (see, for example, Ferry 1992; Keltjens and Vogels 1993; Blaut 1994).

Methyltransferases in acetogenic bacteria. Methyltransferases play an important role in the degradation of C_1-substrates by acetogenic bacteria. These obligately anaerobic bacteria, synthesizing acetyl-CoA for conservation of energy and growth, usually form acetate as main end product (Fig. 5). The methyl group of acetate is formed from CO_2, via formate, formyl-THP, methenyl-THP, methylene-THP, and methyl-THP. The formation of the carboxyl group is catalyzed by the key enzyme in this pathway, CO dehydrogenase. CO is synthesized from CO_2 by CO dehydrogenase which also catalyzes the formation of acetyl-CoA from the methyl group and CO (Fig. 5). Acetyl-CoA is then converted either to acetate in catabolism or used for biosynthesis (see for a review, Diekert and Wolfarth 1994). Homoacetogenic bacteria can utilize different methylated compounds. Examples of such substrates are methanol, methoxylated aromatic compounds such as vanillate or syringate (Diekert and Wohlfarth 1994), and glycine betaine (see, for example, Heijthuijsen and Hansen 1990). Methyltransferases are important enzymes in the degradation of these substrates. A number of experiments with cell extracts of acetogenic bacteria showed that THF served as a methyl acceptor in demethylation reactions (Table 1). It was demonstrated for *Acetobacterium woodii*, grown on phenyl methylethers, that THF and ATP were necessary for the oxygen sensitive *O*-demethylation (Table 1; Berman and Frazer 1992). Activity measured in extracts of syringate-grown *Clostridium thermoaceticum* was found to be relatively oxygen insensitive and reductive activation, by, for example, titanium(III)-nitrilotriacetic acid, was not required. Propyl iodide is known to inhibit proteins containing a corrinoid, but inhibition of the *O*-demethylase by propyl iodide was not observed (Kasmi et al. 1994). The involvement of corrinoids in these reactions can be demonstrated by the light-reversible inhibition by propyl iodide (Brot and Weissbach 1965). In cell fractions of Sporomusa ovata, grown on methanol or 3,4-dimethoxybenzoate, activities were very low (2.0-3.3 $nmol.min^{-1}.mg^{-1}$ protein) and oxygen insensitive (Stupperich and Konle 1993). For measuring the *O*-demethylating activity of *Holophaga foetida*, a new coupled photometric assay was developed (Kreft and Schink 1994; Kreft and Schink 1997). In this assay the NADPH-consuming phloroglucinol reductase reaction was coupled to the phloroglucinol-yielding demethylation of 3,5-dihydroxyanisole. In this way it is possible to study the kinetics of the indicator enzyme phloroglucinol reductase, because phloroglucinol starts to accumulate until it is removed by phloroglucinol reductase at the rate of its production. THF methylation in this organism is probably catalyzed by a multi component enzyme system (Kreft and Schink 1994).

For *Acetobacterium dehalogenans* (formerly known as strain MC; Kaufman et al. 1998) a comparable coupled enzyme assay was developed to measure methyl chloride demethylation and *O*-demethylation (Meβmer et al. 1996). This assay is based on quantification of methyl-THF by a set of three indicator enzymes. First reaction is the methylation of THF by the *O*-demethylase or methyl chloride dehalogenase:

1. $CH_3\text{-}X + THF \rightarrow CH_3\text{-}THF + XH$
   (*O*-demethylase or methyl chloride dehalogenase)

Subsequentely, the methyl-THF is converted to methylene-THF by an NAD^+-dependent methylene-THF reductase:

2. $CH_3\text{-}THF + NAD^+ \rightarrow CH_2{=}THF + NADH + H^+$
   (methylene-THF reductase)

Table 1 Comparison of methyl transfer reactions in cell extracts of the sulfate-reducing bacterium strain WN and acetogenic bacteria

Organism	Substrate	Propyliodide Inhibition	ATP required	Oxygen sensitive	Activity (mU[a] /mg protein)	Substrate specificity	Reference
Strain WN	DMSP	Yes	No	Yes	560	ND	Chapter 4
Eubacterium limosum str PM31	DMSP	ND[b]	No	ND	10.4	ND	Chapter 3
Acetobacterium woodii	PME[c]	Yes	Yes	Yes	14.2	ND	Berman & Frazer (1992)
Clostridium thermoaceticum	Syringate	No	±	No	15	Broad	Kasmi et al. (1994)
Sporomusa ovata	Methanol	Yes	Yes	No	2.0	ND	Stupperich & Konle (1993)
	3,4-DMB[d]	Yes	Yes	No	3.3	ND	
Holophaga foetida	PME	Yes	Yes	ND	81-105	Broad	Kreft & Schink (1994, 1997)
Acetobacterium dehalogenans	Methyl chloride	No	Yes	ND	20	ND	Meβmer et al. (1993, 1996)

[a]: one Unit is defined as µmol/min; [b] : Not Determined; [c]: Phenylmethylethers; [d]: dimethoxybenzoate

The methylene-THF is converted to methenyl-THF by the $NADP^+$-dependent methylene-THF dehydrogenase:

3. CH_2=THF + $NADP^+ \rightarrow$ CH≡THF^+ + NADPH
   (methylene-THF dehydrogenase)

Lactate dehydrogenase was required to drive the thermodynamically unfavorable reaction with methylene-THF reductase:

4. $Pyruvate^- + NADH + H^+ \rightarrow lactate^- + NAD^+$
   (lactate dehydrogenase)

In this way it is possible to measure the formation of NADPH and methenyl-THF photometrically. The advantage of this method is, besides the possibility to do kinetic experiments, that it can be used for all THF-dependent reactions. Using this assay, the group of Diekert succeeded in purifying the ether-cleaving enzyme system, *O*-demethylase, from *A. dehalogenans* (Kaufman et al. 1997). This *O*-demethylase consisted of four different components that all were required for catalysis of the transfer of the methyl group from phenyl methyl ethers to THF. Component B (methyltransferase I) mediated the conversion of the substrate to the reduced corrinoid protein component A. Component D functions as the methylcorrinoid:THF methyltransferase (methyltransferase II). Component C probably functions as an activating protein, reversing oxidation of the protein-bound cobalamin in the presence of ATP and reducing equivalents supplied by the enzymatic oxidation of hydrogen (Kaufman et al. 1998).

Aim and outline of this thesis

The first aim of this thesis project was to identify microorganisms that have the ability to demethylate the algal osmolyte DMSP to the flavor precursors MTPA or mercaptopropionate. This was done by isolating new strains and by screening of culture collections for potential candidates. The second goal was to study the biochemistry of the demethylation reaction of DMSP to MTPA. At the start of this project, a marine sulfate-reducing bacterium, *Desulfobacterium* strain PM4, was known to demethylate DMSP to MTPA (van der Maarel et al. 1993). Other closely related marine sulfate reducers also catalyzed this demethylation reaction (van der Maarel et al. 1996b). This demonstrated the possibility to use microorganisms for the production of MTPA from DMSP in a natural way.

Chapter 2 focuses on aerobic DMSP demethylation. A new isolate belonging to the α-Proteobacteria was found to be able to demethylate DMSP to MTPA. The MTPA was secreted, but subsequently degraded further with a transient accumulation of methanethiol and with dimethyldisulfide as sulfur-containing end product. The closest relative of the isolate, *Ruegeria atlantica*, was also able to demethylate DMSP.

Chapter 3 describes the ability of several acetogenic bacteria to demethylate DMSP stoichiometrically to MTPA. The conversion did not result in any significant increase in biomass. An N-containing structural analog of DMSP, glycine betaine, did support good growth of these organisms.

The sulfate-reducing bacteria that are able to demethylate DMSP (*Db. niacini*, *Db. vacuolatum*, strain PM4 and strain WN) all use the oxidative acetyl-CoA/CO dehydrogenase pathway. It was hypothesized that these bacteria channel the methyl group to the methyl branch of this pathway (Fig. 3; van der Maarel et al. 1996b). Indeed, when cell extracts of DMSP-grown sulfate-reducing bacteria were incubated under anaerobic conditions with THF as a methyl acceptor, MTPA and methyltetrahydrofolate formation was detected (chapter 4). These bacteria also demethylated glycine betaine to dimethylglycine, but cell extracts of DMSP- or glycine betaine grown bacteria did not catalyze the demethylation of glycine betaine with THF as a methyl acceptor.

The demethylation reaction was studied in more detail in chapter 5. In this chapter the purification and characterization of an enzyme specifically catalyzing the demethylation of DMSP (DMSP:tetrahydrofolate methyltransferase) of one of the sulfate-reducing bacteria (strain WN) is described.

Thus far, no anaerobic microorganisms have been isolated that are able to demethylate DMSP completely to mercaptopropionate. Furthermore, the anaerobic demethylation of MTPA to mercaptopropionate by pure cultures of microorganisms was never reported. Chapter 6 shows that under certain conditions the demethylation of MTPA to mercaptopropionate can be demonstrated in sediment suspensions. Organisms responsible for this reaction were methanogenic archaea. With pure cultures of marine *Methanosarcina* strains we showed that MTPA was a good and novel substrate for these archaea.

Finally, the results described in these chapters are discussed and summarized (chapter 7).